Comparison of the Toxic Effects of Hydrogen Peroxide and Ozone on Cultured Human Bronchial Epithelial Cells

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In this study, we compared the cytotoxic and genotoxic effects of hydrogen peroxide and ozone on cultured human airway epithelial cells in primary culture. Both agents caused a dose-dependent loss in the replicative ability of epithelial cells and at higher levels of exposure caused acute cytotoxicity as measured by release of lactate dehydrogenase. Differences were seen, however, between the agents' effects with regard to induction of DNA single-strand breaks as measured by alkaline elution: whereas single-strand breaks were detected in significant amounts at concentrations of hydrogen peroxide that caused acute cytotoxicity, none were detected at any of the levels of ozone exposure examined. A difference was also seen in the ability of the iron chelator deferoxamine to protect cells from the effects of the two oxidants. Preincubation of cultures with deferoxamine appreciably attenuated the toxicity of hydrogen peroxide but not of ozone. These data suggest that ozone has significant toxic effects on bronchial epithelial cells not mediated through the generation of hydrogen peroxide or hydroxyl radical. Furthermore, the data indicate that the inhibiting action of ozone on cell replicative ability is not mediated through a mechanism related to DNA single-strand breaks. *Key words*: cell replication, cytotoxicity, deferoxamine, hydrogen peroxide, ozone, single-strand breaks. *Environ Health Perspect* 102:972–974 (1994)

Ozone is one of the most important toxic components of photochemical smog, and exposures of humans to ozone have been demonstrated to induce airway inflammation and affect airway reactivity (*1,2*). Because ozone is extremely reactive, it has been considered highly unlikely that cells other than those within the airways or airway epithelial cells would be directly exposed to ozone, and secondary intermediates, such as hydrogen peroxide or aldehydes, may be important mediators of the biotoxic effects of ozone.

Recent experiments in our laboratory have demonstrated that airway epithelial cells exhibit a transient increase in permeability (*3*) after low levels of ozone exposure. The mechanisms of injury and recovery of the epithelial cell barrier are of particular interest because epithelial cells may be important modulators of access for antigen and other airborne agents that could potentially react with mucosal and submucosal cells. Identification of intermediate reactive species of ozone and possible scavengers of those intermediates could have implications for preventing or attenuating the effects of ozone on airway reactivity.

In this study, we investigated the possibility that the toxic effects of ozone on bronchial epithelial cell cultures are a result of a hydrogen peroxide intermediate. We compared ozone to hydrogen peroxide with regard to ability to induce DNA single-strand breaks and investigated the potential of the iron chelator deferoxamine to abolish or attenuate the toxic effects of the agents *in vitro*.

Methods

Cell culture and viability assays. BEAS2B cells (*4*) were obtained from Curtis Harris at the National Cancer Institute and maintained in KGM medium (Clonetics Corp., San Diego, California). These cells were derived by transfection of an adeno-SV40 hybrid virus into primary bronchial epithelial cells and have retained the ability to differentiate to either a secretory or squamous phenotype. Monolayer cultures of BEAS2B grown on plastic culture plates were exposed to H_2O_2 (Costar, Cambridge, Massachusetts). For exposure to ozone, cells were grown to monolayer confluence on 0.4-μm pore size Transwell polycarbonate filters (Costar), and medium above filters was removed 2 days before exposure. This resulted in a layer of cells at a gas–fluid interface with only a thin film of fluid above the cells. Before exposures we removed medium, rinsed cultures three times, and exposed cells in Hanks' Balanced Salt Solution (HBSS). For experiments investigating the effect of iron chelation on toxicity, cultures were incubated with deferoxamine (CIBA) at a concentration of 2.5 mM in HBSS for 30 min before exposure. We then removed the deferoxamine-containing buffer and again rinsed cultures three times with HBSS immediately before exposure. After exposures were completed, we removed buffer and replaced it with KGM medium above and below filters.

We determined release of lactate dehydrogenase (LDH) by cultures by measuring an increase in absorbance at 340 nm after addition of NAD. In previous experiments (data not shown), we determined that significant release of LDH by oxidant-injured cells occurred during the 2 hr after exposure as well as during the exposure; therefore, in these experiments we totaled the amounts of LDH in the exposure buffer and in the media 2 hr after the termination of exposure to ozone or hydrogen peroxide and expressed this number as a percentage of total release by cultures exposed to 1% Triton-X 100. Colony-forming efficiency was determined by subculturing cells 2–4 hr after exposure at a density of 2000 cells per duplicate culture plate and allowing growth for 8–10 days. Numbers of colonies (defined by an aggregate of at least 20 cells) were counted and standardized to numbers of colonies formed by unexposed cells. For these experiments, exposures to ozone or hydrogen peroxide were conducted in triplicate, and the same cultures were used for both determination of LDH release and colony-forming efficiency.

Ozone exposure system. Confluent BEAS2B cultures on polycarbonate filters were exposed for 2 hr or 4 hr at 37°C in matched 6-l plexiglass chambers to 5% CO_2/95% air (control) or 5% CO_2/95% air containing specific concentrations of ozone. The flow rate in each chamber was maintained at 1 l/min, and ozone was generated by passing the in-flow to one chamber past a UV mercury vapor lamp. The ozone concentration in that chamber was continuously monitored with a Mast 724 meter and was regulated by variations in the UV lamp intensity. The in-flow to each chamber was bubbled through water to achieve 97% relative humidity. We allowed the ozone chamber to equilibrate for at least 1 hr at the selected ozone concentration before adding the cells. We began timing the exposure period when the chamber concentration was reestablished after adding the plates containing cells (generally 30 min).

Measurement of DNA damage. To measure DNA single-strand breaks, we prelabeled cells with ^{3}H-thymidine, 10 μCi/mM, for 24–48 hr before exposure to agents. At the termination of exposure, cells were rinsed, scraped off the dishes in chilled HBSS, and placed onto filters for the alkaline elution procedure of Kohn et al. (*5*). In this method, an alkaline buffer denatures DNA to the single-stranded form, and DNA is eluted at a rate of 2 ml/hr through a nonbinding polycarbonate filter. Single-strand breaks are quanti-

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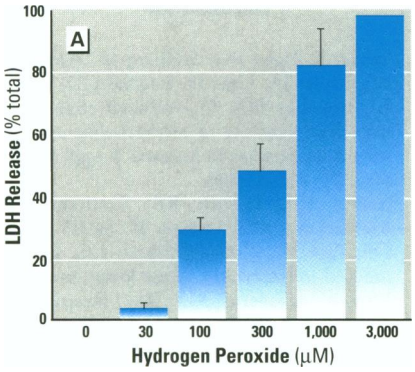


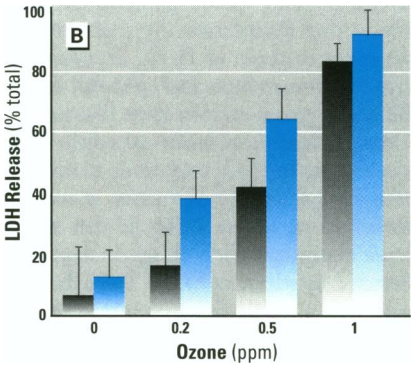


Figure 1. Lactate dehydrogenase (LDH) release by BEAS2B cultures exposed to (A) H_2O_2 for 1 hr or (B) ozone for 2 hr (gray bars) or 4 hr (blue bars). The LDH measured at completions of exposures and 2 hr after termination of exposures were totaled. Values represent means of two experiments, each with triplicate exposures, and error bars represent SEMs. Release by cultures exposed to 100 μM H_2O_2 is significant ($p < 0.01$) and for 0.5 ppm ozone for 2 hr or 0.2 ppm ozone for 4 hr ($p < 0.05$).

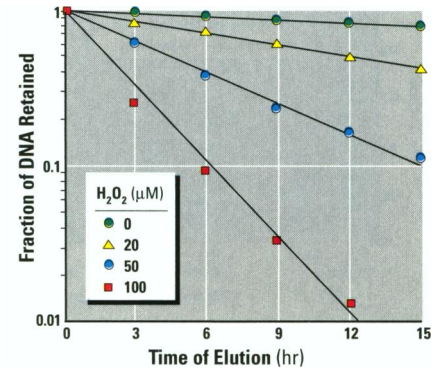


Figure 2. DNA single-strand breaks induced by exposure of cell cultures to 0, 20, 50, or 100 μM H_2O_2 for 1 hr. The fraction of DNA retained on the filter was determined by counting radioactivity eluted at 3-hr intervals. Less than 5% variablity was seen in duplicate measurements. No single-strand breaks were detected in ozone-exposed cultures (i.e., fraction of DNA retained was within 5% of control).

fied by scintillation detection of radiolabeled DNA in fractions that are collected at 3-hr intervals.

Results

Measurements of LDH were made at the end of exposures and 2 hr after termination of exposures. Total release was determined by exposing control cultures to 1% Triton-X 100 for 20 min and measuring LDH activity in supernatant. In cultures exposed to H_2O_2, significant release of LDH was observed at concentrations as low as 100 μM. Exposures to ozone also generated release of LDH which appeared to be time and dose dependent; significant release was seen at 0.5 ppm ozone for 2 hr or 0.2 ppm ozone for 4 hr (Fig. 1).

Hydrogen peroxide is well known to cause DNA single-strand breaks either as direct strand breaks or as base damage (such as formation of thymine glycols) which become alkali-labile sites. In our cell cultures, a concentration-dependent relationship between H_2O_2 exposure and induction of DNA single-strand breaks was observed (Fig. 2). The level of DNA strand breaks seen after 100 μM exposure for 1 hr was approximately equivalent to 300 rad γ-irradiation damage. No significant DNA single-strand breaks were detected at concentrations of H_2O_2< 20 mM or at any of the ozone exposures examined (DNA retained on filter was not significantly different from control).

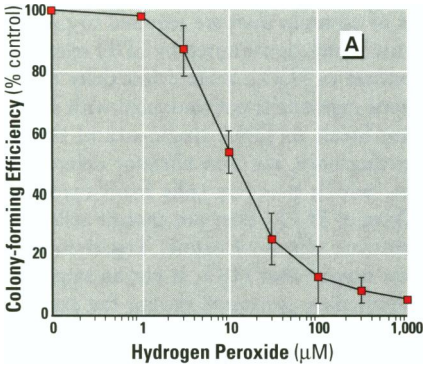
In experiments on replicative ability, cultures were exposed to hydrogen peroxide for 1 hr or to ozone for 2 hr and 4 hr, and replanted cells were allowed to replicate for a sufficient period of time to produce colonies. The exposures were performed at a cell density of 1–5 × 10^4 cells/cm^2. A concentration-dependent loss of colony-forming efficiency was observed after H_2O_2 exposure, with a 50% loss observed at approximately 10 μM H_2O_2 (Fig. 3A). A 50% loss in colony-forming efficiency was observed after exposure to 0.5 ppm ozone for 2 hr or 0.2 ppm ozone for 4 hr (Fig. 3B). For both exposures, some survival was observed at relatively high concentrations. Thus, loss of replicative ability was observed at lower levels of exposure to both agents than was release of LDH.

Preincubation of cultures with deferoxamine before H_2O_2 exposure has been previously described to have protective effects as a result of iron chelation (*6*). These protective effects were also observed in our cell cultures preincubated with 2.5 mM deferoxamine for 30 min before H_2O_2 exposure. Marked decreases in the levels of DNA single-strand breaks, decreases in release of LDH, and increases in replicative ability were all observed in H_2O_2-exposed cultures preincubated with deferoxamine when compared to those not preincubated with deferoxamine (Fig. 4). In contrast, preincubation with deferoxamine did not appear to have significant protective effects on the cell cultures exposed to ozone (i.e., preincubation with deferoxamine did not affect LDH release nor ability of cells to replicate after exposure to ozone).

Discussion

Although ozone has been identified as an important toxic agent to the respiratory and immune systems, the mechanisms of its cellular toxicity remain poorly defined. Because ozone is extremely reactive and unstable, it has been postulated that the mechanism of ozone toxicity is related to generation of secondary toxic products, particularly hydrogen peroxide and aldehydes (*7*).

Several recent experiments support the role of such secondary products in mediating the toxicity of ozone. For example, dioleoyl phosphatidylcholine liposomes were ozonated and found to generate 1 mol-equiv H_2O_2 and 2 mol-equiv aldehydes, based on moles of ozone consumed (*8*). The ozonated liposome mixture induced red blood cell hemolysis, which was partially protected by antioxidants such as ascorbate, catalase, and glutathione. These experiments suggested that



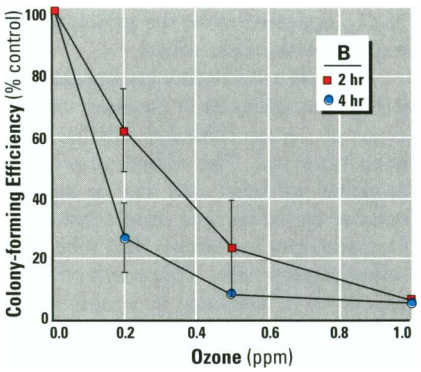


Figure 3. Colony-forming efficiency (CFE) by BEAS2B cells exposed to (A) H_2O_2 for 1 hr or (B) ozone for 2 hr or 4 hr. The same cultures exposed for experiments illustrated in Figure 1 were subcultured at clonal density to determine colony-forming efficiency. Decrease of CFE is significant for cultures exposed to 10 μM H_2O_2 ($p < 0.01$), for cultures exposed to 0.2 ppm ozone for 2 hr ($p < 0.05$), and for cultures exposed to 0.2 ppm ozone for 4 hr ($p < 0.01$).

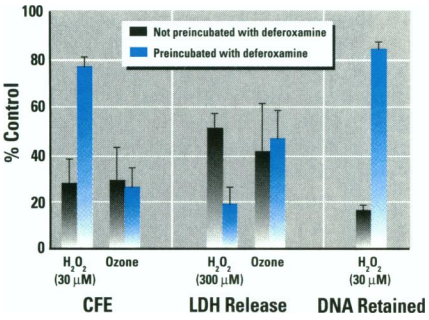


Figure 4. Effect of preincubation with deferoxamine on toxic effects of H_2O_2 or 0.5 ppm ozone for 2 hr. Values are means + SEM. Control cultures for colony-forming efficiency (CFE) and DNA retained were not exposed to any agents; control cultures for lactate dehydrogenase (LDH) release were treated with 1% Trition-X 100 for 30 min. Protective effects of deferoxamine for CFE and LDH release in cultures exposed to H_2O_2 is significant ($p < 0.01$).

both H_2O_2 and aldehydes were generated as cytotoxic species from the ozonation of unsaturated fatty acids. In other experiments (*9*), ozone-exposed aqueous solutions of the tobacco-smoke-derived arylamine 1-naphthylamine produced hydroperoxides and a DNA-damaging product (to cultured lung cells) that was not eliminated by treatment with catalase. These authors concluded that neither H_2O_2 nor superoxide anions were involved in breaking DNA (*9*). In other experiments, arachidonic acid was exposed to ozone and found to degrade to predominantly aldehydic substances and H_2O_2 (*10*). Similar aldehydic substances were produced by human bronchial epithelial cells (BEAS2B) exposed *in vitro* to ozone. *In vitro*, these arachidonic acid products induced an increase in human peripheral blood polymorphonuclear leukocyte polarization, decreased human peripheral blood T-lymphocyte proliferation in response to mitogens, and decreased human peripheral blood natural killer cell lysis of K562 target cells. The aldehydic substances, but not H_2O_2, appeared to be the principal agents responsible for these effects.

The role of secondary intermediates such as H_2O_2 or aldehydes in the direct toxicity of ozone on airway epithelial cells has not yet been reported. The effects of ozone on bronchial epithelial cells may be important because epithelial cells form a barrier that blocks antigen interaction with subepithelial inflammatory cells. This barrier function appears to be disrupted by ozone exposure, based on our recent experiments that demonstrated a decrease in electrical resistance and increased permeability to mannitol in a canine airway epithelial cell layer *in vitro* (*3*).

Our approach to investigating the possible role of H_2O_2 in mediating the toxic effects of ozone on the epithelial cell barrier was to directly compare the effects of ozone exposure to those of H_2O_2 exposure on a cultured human bronchial epithelial cell line, BEAS2B, grown on micropore filter surfaces. Characterization of bronchial epithelial cell cytotoxicity to H_2O_2 and ozone in this study revealed that significant toxicity, as measured by loss of replicative ability, is seen at lower levels of exposure for both agents than can be detected by release of LDH. These data indicate that a subtle form of injury that can impair replicative ability but that cannot cause cytolysis occurs at these low levels. The effects on replicative ability of cells may have important implications for the ability of cells and tissues to repair initial damage.

The mechanisms of H_2O_2 toxicity are not yet completely understood, particularly with regard to its effects on cell replicative ability. Cytolysis of cells exposed to H_2O_2 occurs at levels of exposure that are associated with significant DNA single-strand breaks, and some authors have suggested that this cytolysis is a result of depletion of cellular NAD and ATP resulting from attempted repair of the DNA damage (*11,12*). This explanation is not applicable to the loss of replicative ability seen at lower levels of exposure of H_2O_2 that are not accompanied by significant DNA single-strand breaks (*13*), nor any of the toxic effects of ozone, which do not appear to induce DNA single-strand breaks in these cells.

The toxicity of H_2O_2 on biological systems depends on the iron-catalyzed conversion of H_2O_2 to hydroxyl radical (*6,14*). This reaction can be blocked by an iron chelator, such as deferoxamine. In our study, deferoxamine markedly decreased all toxic effects of H_2O_2 on bronchial epithelial cells, as expected. Deferoxamine did not have a significant effect, however, on the acute cytotoxic effects or loss of replicative ability caused by exposure of the cells to ozone.

In summary, ozone exposure causes decreased replicative ability in cultures of human bronchial epithelial cells at lower levels of exposure than are required to produce acute injury, as measured by LDH release. In contrast to H_2O_2, acute cytotoxicity due to ozone exposure is not associated with significant levels of DNA single-strand breaks. Furthermore, the iron chelator deferoxamine, which protects cells from cytotoxic effects of H_2O_2, does not protect cells from cytotoxic effects of ozone. Together, these data suggest that H_2O_2 is not an important intermediate generated during the exposure of bronchial epithelial cells to ozone and that other reactive species, such as aldehydes, deserve consideration for their importance in mediating the effects of ozone on bronchial cells.

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